

# Enzymatic Degradation: Effects of Degradation Time on the Physical Characteristics and Water Solubility Properties of Chitosan

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## ABSTRACT:

**Introduction:** Chitosan (poly- $\beta$ -(1-4)-D-glucosamine) is a linear polysaccharide polymer known as a potential bioactive material that is useful in pharmaceutical applications. This is due to its bioadhesive, antimicrobial, permeability enhancing and biodegradable properties. However, since it is poorly soluble under physiological conditions, this drawback makes chitosan difficult to formulate and utilize in biological applications.

**Methods and Results:** In this study, we optimized the hemicellulase enzymatic hydrolysis of medium molecular weight chitosan (MWCS) by varying the reaction time: 2 hours, 4 hours and 6 hours. The resulting chitosan was characterized for the change in its physical characteristics using Fourier transform infra-red (FTIR), x-ray diffraction (XRD), contact angle analyses and scanning electron microscopy (SEM). FTIR results showed no significant changes in the functional groups present in the chitosan after enzymatic degradation. XRD diffractograms depict changes of chitosan internal crystal structure from amorphous to crystalline, which improves its stability. SEM images showed an increase in smoothness on the chitosan's surface morphologies. The surface contact angle analysis showed reduced contact angle as the reaction time increased and this results in the improvement of wetting properties of medium molecular weight chitosan, hence improved water solubility.

**Conclusions:** In conclusion, as the duration of enzymatic hydrolysis increased, the water solubility properties, as well as other physical and chemical properties of low molecular weight chitosan was also improved.

**Key words:** Chitosan; Hemicellulase; Enzymatic degradation; Drug Delivery; Solubility

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## 1. Introduction

Chitosan is a cationic natural polymer with a complete amino group, obtained by deacetylation of chitin with alkaline solution [1]. This process results in removal of some acetyl groups from the molecular chain and shortened the chain lengths of the chitin, [2]. When the degree of deacetylation reaches 50%, it spontaneously becomes soluble in aqueous acidic media. Solubilisation process follows or develops by the protonation of  $-NH_2$  group on the carbon 2 position of the D-glucosamine unit, and the polysaccharide is converted to a polyelectrolyte in the medium [3].

Chitosan's intrinsic properties such as biodegradability, biocompatibility, special adhering feature to the mucosal surface, penetration enhancing by opening epithelial tight-junctions, immune-stimulating activity and antimicrobial properties have drawn attention exploiting chitosan as drug delivery systems and antimicrobial agents [4,5]. Even though chitosan is known to have

important functional activities, the poor solubility of chitosan is the major limiting factor in its utilization. This interferes with the biomedical application of chitosan, especially at the physiological pH value (7.4) where chitosan is insoluble and ineffective as an absorption enhancer [6]. Hence, improving the solubility of chitosan is crucial for its utilization in a wide pH range. New interest has recently emerged on partially hydrolyzed chitosan in which the molecular weight of chitosan decreases, hence making it readily soluble in water due to its shorter chain length and free amino groups in D-glucosamine units [7,8]. The low viscosity and greater solubility of hydrolyzed chitosan at neutral pH have attracted the interest of many researchers to utilize chitosan in its lower molecular weight form [9].

In this study, we used the enzymatic degradation method to synthesise water-soluble chitosan from MWCS. We optimized the hemicellulase enzymatic hydrolysis of low molecular weight chitosan by varying the reaction time: 2 hours, 4 hours and 6 hours. The effects of this

enzymatic method on the physical characteristics of chitosan were investigated using Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) analysis, contact angle measurement, and scanning electron microscopic (SEM) analysis.

## 2. Materials & Methods

The experiment design for this study is summarized in Figure 1.

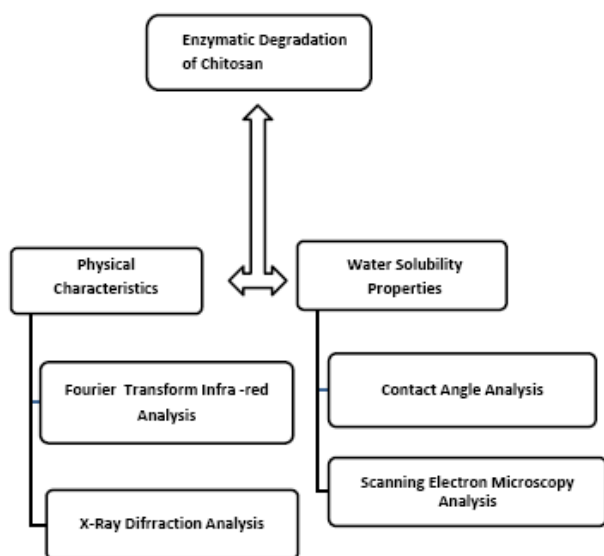


Figure 1: Experiment design

### 2.1. Preparation of Water Soluble Chitosan

The preparation of water-soluble chitosan was modified from the method reported by Qin et al., 2002 [10]. MWCS (1g) was completely dissolved in 50 mL of 2% v/v acetic acid. After stirring for 3 hours, the pH of the mixture was adjusted to the desired pH range with 1M sodium hydroxide (NaOH) or hydrochloric acid (HCl) and left overnight. HCl was used instead of acetic acid to avoid changing the initial concentration of acetic acid. The mixture was then placed in a water bath at a specified temperature. A solution containing 20% w/v of hemicellulase enzyme was added to initiate the reaction. The mixture was left to react with the hemicellulase enzyme for three different reaction times which were 2 hours, 4 hours and 6 hours. Then, the mixture was removed from the water bath and the pH adjusted to 5.5 with 1M NaOH to neutralize the mixture. The mixture was then boiled for 10 min to denature the hemicellulase enzyme. The denatured enzyme on the upper layer of the mixture was separated from the solution and removed using a spatula. The mixture was concentrated to about 1/5 using a rotary evaporator at temperature 60°C and freeze-dried to obtain water-soluble chitosan.

### 2.2. FTIR Analysis

FTIR spectra of the chitosan films were collected by using a Fourier Transform Infrared Spectrometer that was operating in the range of 4,000 - 400  $\text{cm}^{-1}$  at a resolution of 4  $\text{cm}^{-1}$ . Chitosan and water-soluble chitosan

samples were prepared in the form of potassium bromide (KBr) discs. Sample powder (1mg) and KBr (69mg) were blended and triturated by using a mortar and pestle for approximately 3 minutes to form a mixture. The mixture (70mg) was compacted at a pressure of 10 tons for 1 minute by using an IR hydraulic press.

### 2.3. XRD Analysis

Wide-angle XRD patterns were obtained using a Bruck D8 Advance powder diffractometer with Cu K $\alpha$  radiation ( $\lambda = 0.154 \text{ nm}$ , 40 kV, 30 mA) and Bragg-Brentano ( $\theta$ ,  $2\theta$ ) geometry at room temperature. The Wide-Angle-X-Ray diffraction measurements of the sample were taken and the scanning speed was 0.1 s/step with a step length of 0.01° [11].

### 2.4. Contact Angle Analysis

The contact angle measurements were conducted by performing the sessile drop method with a static contact angle instrument: (OCA 15 EC, Data physics, Germany). The equipment's part consists of a horizontal stage to mount a liquid or solid sample, a micrometer pipette to form a liquid drop, an illumination source and a telescope with a protractor eyepiece. The measurement of the contact angle was achieved by reading the protractor through the eyepiece after aligning the tangent of the sessile drop profile at the contact point of the surface [12]. Before the procedure, a hydraulic press with a maintained specific pressure was used to compress the samples to form a disk. To calculate the contact angle, a drop of liquid which was pure water, was dispensed onto the sample surface and the contact angle was calculated by using a mathematical expression known as half angle method ( $\theta/2$  method). The half-angle method was applied from the droplet shape in which by applying the tangent line from the contact point along with the liquid-vapor interface in the droplet profile. The procedure was conducted under ambient air conditions of 25°C  $\pm$  2°C or 55°C  $\pm$  5°C and the contact angle measurements were calculated automatically.

### 2.5. Morphology Studies

The chitosan morphology was verified using SEM. Chitosan samples were coated with silver and viewed under SEM with magnifications from 200 to 10000 times with spatial resolution of 50 to 100 nm in order to observe the external morphology or texture of chitosan.

## 3. Results

### 3.1. FTIR Analysis

Table 1 summarises the FTIR functional groups of chitosan samples before and after enzymatic degradation. It can be observed that the chitosan samples retain their functional groups after enzymatic degradation. Therefore, we can conclude that hemicellulase enzymatic hydrolysis is a suitable method to improve solubility of MWCS without affecting the structure of MWCS.

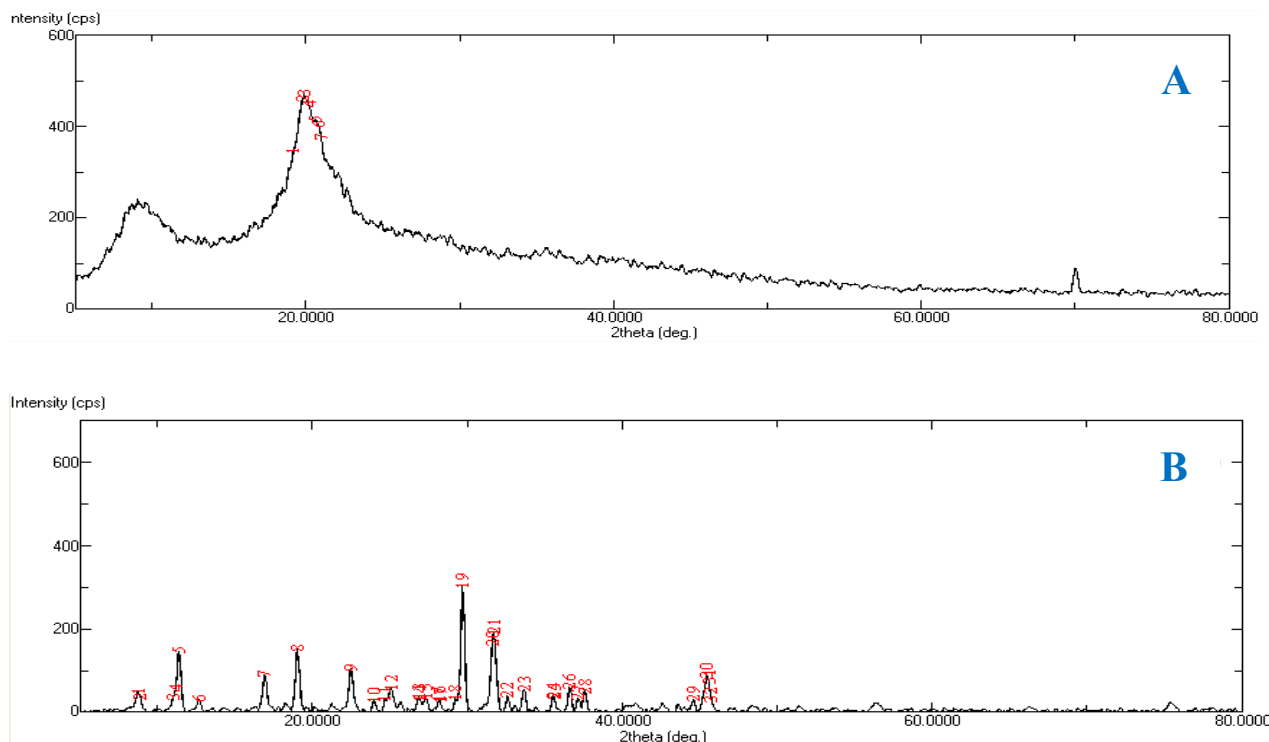
**Table 1:** Summary of functional groups of MWCS (before and after enzymatic degradation) using FTIR.

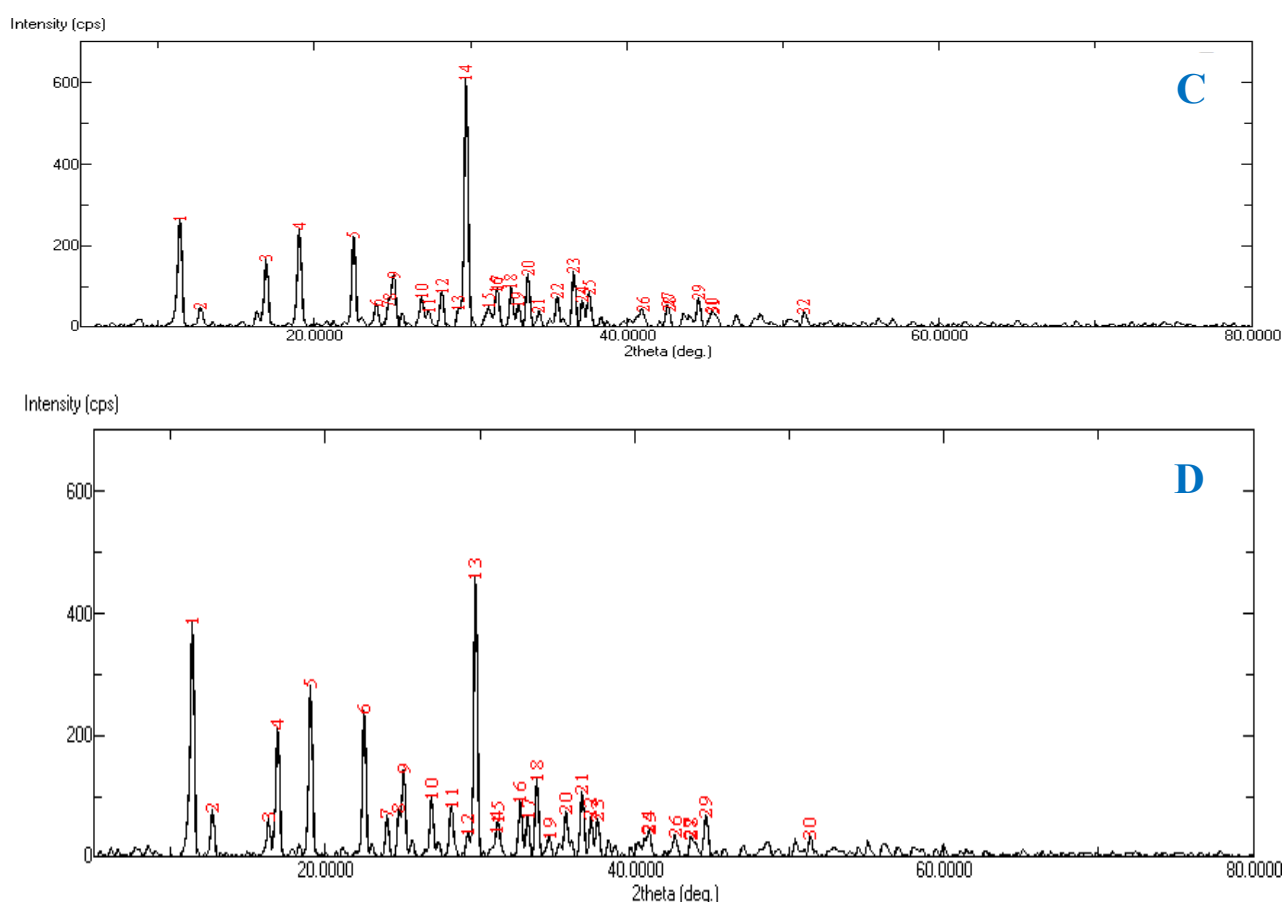
Functional Groups	Peak frequency (cm <sup>-1</sup> )				
	References	MWCS	MWCS(2hours)	MWCS(4hours)	MWCS(6hours)
O-H stretch Hydroxyl	3590-3650 <sup>[15]</sup> , 3450 <sup>[14]</sup>	Overlapping with N-H stretch	3644	Overlapping with N-H stretch	Overlapping with N-H stretch
N-H stretch Amine	3500-3300 <sup>[17]</sup> , 3350 <sup>b-[14]</sup>	3407	3442	3439	3433
Amide I CONH <sub>2</sub> (C=O stretch)	1680-1630 <sup>[17]</sup> , 1600-1670 <sup>[16]</sup> , 1655 <sup>[10]</sup>	1655	1645	1642	1641
Amide II CONH (NH bending)	1600-1640 <sup>[18]</sup>	Overlapping with Amide I	Overlapping with Amide I	Overlapping with Amide I	Overlapping with Amide I
Amide III	1320 <sup>[14]</sup> , 1380 <sup>[18]</sup> , 1321 <sup>[10]</sup>	1320	1346	1340	1341
Primary Amine NH <sub>2</sub> bending	1650-1560 <sup>[14]</sup> , 1600 <sup>[10]</sup> , 1590 <sup>[17]</sup>	1570	1562	1561	1560
C-O-C bridge stretch	1170-1050 <sup>[10]</sup>	1079	1077	1078	1078
C-O stretch vibration	1150-1040 <sup>[15]</sup>	Overlapping with C-O-C	1152	1153	1152

### 3.2. XRD Analysis

Figure 2 depicts the XRD diffractograms for all chitosan samples. It can be seen that there are two typical diffraction peaks of MWCS at 2 theta 10° and 2 theta 20°. This peak contributes to the amorphous characteristic of chitosan due to its wide and broad bend in the baseline. However, after enzymatic degradation, the intensities of the peak at 2 theta 10° were increased

as the duration of enzymatic hydrolysis increased. Intensities of a peak can be influenced by two parameters which are the size of the molecules and orientation of particles in the powdered sample. After 6-hour enzymatic degradation, the peak formed at 2 theta 10° shows the highest intensity and this might be due to a more ordered arrangement seen in the crystal structure.





**Figure 2:** XRD diffractograms of (A) MWCS, (B) MWCS after 2 hours' degradation, (C) MWCS after 4 hours' degradation and (D) MWCS after 6 hours' degradation.

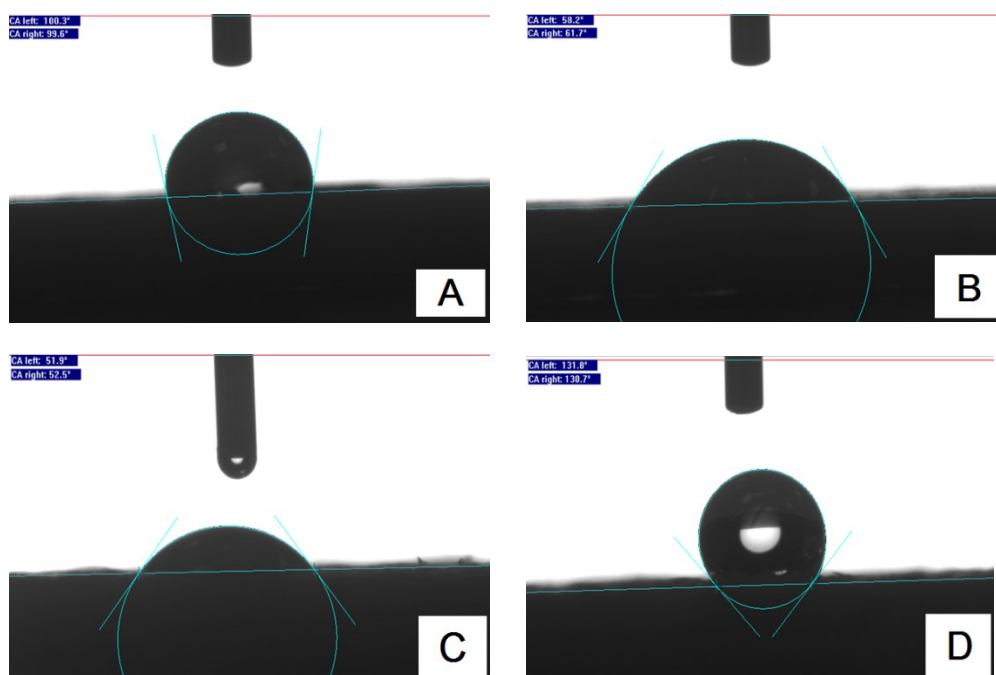
### 3.3. Contact Angle Analysis

According to previous literature, the optimum reaction time needed to hydrolyze medium molecular weight chitosan to a low molecular weight chitosan was 6 hours. Hence, the physical characteristics of chitosan are expected to exhibit the highest water solubility when being hydrolyzed by hemicellulase enzyme for 6 hours. Our studies also concluded that 6 hours' enzymatic hydrolysis MWCS showed the highest wettability as compared to 2 hours and 4 hours enzymatic hydrolysis reaction time (Table 2). Contact angle less than  $90^\circ$

indicates that the wetting of the chitosan surface is favorable meanwhile contact angle more than  $90^\circ$  (as shown by MWCS and 2 hours' degradation time) indicated that wetting of the surface was unfavorable. Figure 3 shows the images of the contact angle measurement of chitosan after 2 hours (A), 4 hours (B), 6 hours (C) degradation and MWCS (D) respectively. After 6 hours' reaction time, MWCS possess hydrophilicity properties as the water tends to bead and wet the chitosan surface.

**Table 2:** Surface contact angle measurement of chitosan samples using water.

Chitosan	Left Contact Angle ( $^\circ$ )	Right Contact Angle ( $^\circ$ )	Average Contact Angle ( $^\circ$ )
A (2 hours degradation)	100.3	99.6	100
B (4 hours degradation)	58.2	61.7	60
C (6 hours degradation)	51.9	52.5	52.2
D (MWCS)	131.8	130.7	131.3

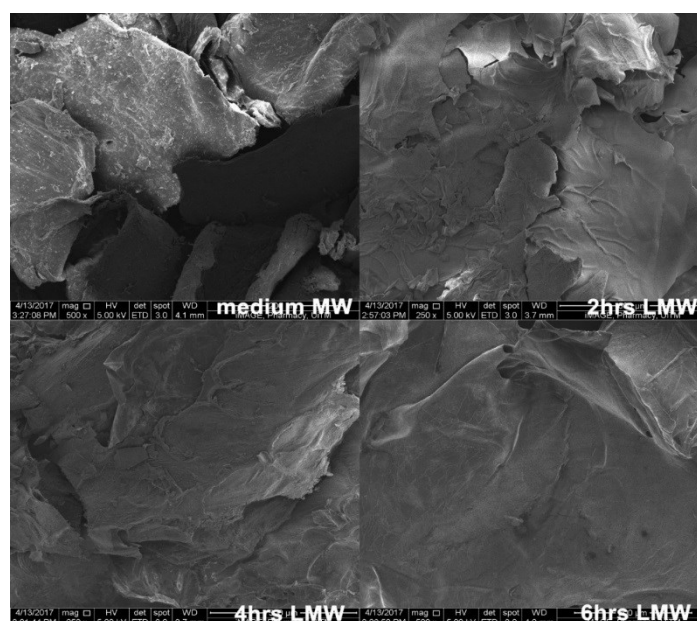


**Figure 3:** Contact angle measurement of MWCS after (A) 2 hours degradation, (B) 4 hours degradation, (C) 6 hours degradation and (D) pure MWCS, using water as the probe liquid.

### 3.4. Morphology Studies

SEM images revealed that MWCS prepared by enzymatic hydrolysis exhibits smoother particles morphology (Figure 4). The distinctive presence of peaks and trough can be seen on the MWCS morphology. MWCS exhibits the highest thickness and less uniform in the particles surface distributions. After 2 and 4 hours enzymatic degradation, it could be observed

that the thickness and uniformity of chitosan surface were decreased until they were seen to achieve almost a complete uniformity of morphology after 6 hours enzymatic degradation. The roughness of chitosan surface might be due to distinctly arranged microfibrillar crystalline structure that could be degraded during hemicellulase-mediated hydrolysis of chitosan [13], exposing the true surface for dissolution.



**Figure 4:** SEM images of chitosan samples' morphology (10 000 magnifications).



#### 4. Discussion and Conclusion

Lower molecular weight chitosan prepared by hemicellulase enzymatic degradation of MWCS does exhibit improvement in its water solubility, stability and better surface morphology while maintaining its major chemical structure. FTIR studies of the chitosan samples showed the backbone structure of chitosan which is responsible for most of the chitosan's activity was maintained after degradation. XRD analysis obtained showed the changes in chitosan internal structure from an amorphous to a more crystalline structure after degradation, hence improving the stability of chitosan. The surface contact angle measurements showed improvement in the wetting properties of MWCS after enzymatic degradation. SEM analysis of the chitosan samples exhibited an improvement in the smoothness and distribution of particles on the chitosan surface as the duration of enzymatic hydrolysis of chitosan was increased. These effects may contribute to the improved water solubility of chitosan. In conclusion, with our findings, hemicellulase enzymatic hydrolysis is a suitable method to improve water-solubility of chitosan without affecting its physicochemical nature and biomedical applications.

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#### Conflict of interest

There is no conflict of interest.

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